

REMARKS

This application has been amended in a manner that is believed to place it in condition for allowance at the time of the next Official Action.

Claims 1-17 are pending in the application. Claims 1, 5-9 and 15 have been amended to address the formal matters raised in the outstanding Official Action. New claims 16-17 have been added. Support for the new claims may be found generally throughout the specification. Applicant respectfully submits that no new matter has been added.

Claims 1-15 were rejected under 35 USC 112, first paragraph, for allegedly not satisfying the written description requirement. This rejection is traversed.

Applicant respectfully submits that the outstanding Official Action fails to satisfy its burden in showing that the claimed invention does not satisfy the written description requirement.

The inquiry into whether the description requirement is met must be determined on a case-by-case basis and is a question of fact. *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976). Moreover, a description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. See, e.g., *In re Marzocchi*, 439 F.2d 220, 224, 169

USPQ 367, 370 (CCPA 1971). The examiner, therefore, has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976).

The Official Action fails to present any evidence to suggest that the claimed invention does not satisfy the description requirement. Accordingly, applicant respectfully requests that the rejection be withdrawn.

Claims 1-15 were rejected under 35 USC 112, first paragraph, for allegedly not satisfying the enablement requirement. This rejection is traversed.

In order to substantiate the claims over their full scope and to show that the claimed subject matter, in particular the method for the therapeutic treatment of lymphoma and leukemia according to current claim 4, is enabled by the patent specification, the applicant presents experimental data showing the effectiveness of o-ATP as antiangiogenic and antiapoptotic agent, using either *in vitro* or *in vivo* models. The Examiner's attention is respectfully directed to the Rule 132 Declaration in the appendix of this amendment.

The antiangiogenic effects of o-ATP were demonstrated in experiments where either non-stimulated or TNF $\alpha$ -stimulated PBMC transendothelial migration was inhibited by the addition of

o-ATP. In a second set of experiments, o-ATP was shown to inhibit cell-proliferation by inducing apoptosis in RMA (lymphoma) cells in culture. Similar results were obtained *in vivo*, where o-ATP proved able to reduce tumor growth in mice injected with RMA cells.

These results are predictive of an antitumor activity, especially against lymphoma and leukemia, as recited in claim 4.

Therefore, in view of the data evidencing that o-ATP is an effective antiangiogenic and antiapoptotic agent *in vitro* and *in vivo* models, the claimed invention is enabled, and withdrawal of the rejection is respectfully requested.

Claims 1, 5 and 9-15 were rejected under 35 USC 112, second paragraph, for allegedly being indefinite. This rejection is traversed.

Claim 1 has been amended as suggested by the Examiner. The applicant thanks the Examiner for the suggestion as how to overcome the rejection.

As to the term o-ATP, applicant submits that the term is definite to one skilled in the art. Indeed, it is believed that the Examiner's skilled explanation of the state of the art shows that one skilled in the art would understand the term. Indeed, while the term may be broad, the term is definite to one skilled in the art.

Applicant also respectfully submits that claim 5 is definite to one skilled in the art. Indeed, one skilled in the art would plainly understand what constitutes cytotoxic compounds, cytostatic compounds, antimetabolites, alkaloids, antibiotics, alkylating agents, peptides, biological response modulators, cytokines, drugs and statins. Furthermore, MPEP §2173.05(h) clearly states that genus, subgenus, and Markush-claims if properly supported by the disclosure, are all acceptable ways for applicants to claim their inventions. It provides different ways to present claims of different scope. Examiners should therefore not reject Markush-type claims merely because they are genus claims and encompass Markush-type claims.

Claims 5-8 have been amended to recite a composition. In that the claims broadly recite a composition, it is not necessary that the claims recite a "pharmaceutically acceptable carrier".

The dependency of claim 15 has been changed as suggested by the Examiner.

Claim 1-4, 9-15 were rejected under 35 USC 102(b) as allegedly being anticipated by CORY et al. Claims 1-4 and 9-15 were rejected under 35 USC 103(a) as allegedly being unpatentable over CORY et al. These rejections are traversed.

The Examiner's attention is respectfully directed to the fact that the Ehrlich tumor (ET) used by CORY et al. is not a lymphoma, but a "low metastatic spontaneously occurring murine

mammary carcinoma" (see the enclosed article published in Experimental Hematology (1999)). Thus, CORY et al. cannot anticipate the claimed invention.

CORY et al. also fail to render obvious the claimed invention. CORY et al. report the study of RNA-synthesis inhibition in ET by the dialdehyde derivative of Inosine, while the dialdehyde derivative of ATP was previously reported to inhibit ribonucleotide reductase activity in cell-free extracts, i.e. in an experimental setting that is not predictive or an antiproliferative activity, still less of an activity against lymphoma or leukemia tumors (CORY et al., page 814, paragraph bridging two columns). In view of the above, one skilled in the art would not find any motivation in CORY et al. to use oxidized ATP for the treatment of VEGF-induced (endothelial) cell proliferation, let alone lymphoma or leukemia.

Thus, applicant respectfully requests that the rejections be withdrawn.

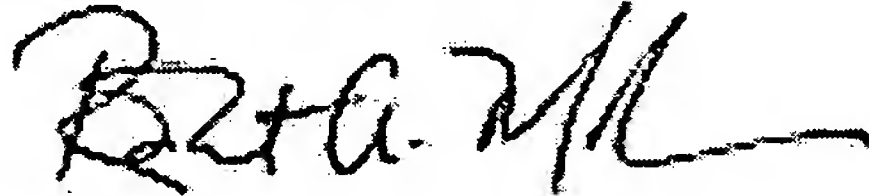
In view of the present amendment and foregoing remarks, therefore, applicant believes that the present application is in condition for allowance at the time of the next Official Action. Allowance and passage to issue on that basis is respectfully requested.

Charge the fee of \$105 for the one independent claim added herewith to our credit card.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

YOUNG & THOMPSON

A handwritten signature in black ink, appearing to read "R.A. Madsen", with a long horizontal flourish extending to the right.

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APPENDIX:

- Jose Ruiz de Morales et al. publication
- Declaration of Maria Elena Ferrero

## Ehrlich tumor stimulates extramedullar hematopoiesis in mice without secreting identifiable colony-stimulating factors and without engagement of host T cells

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(Received 1 December 1998; revised 3 August 1999; accepted 25 August 1999)

Tumor growth is associated with neutrophilia, thrombocytosis, and extramedullar hematopoiesis. The mechanism(s) accounting for these phenomena is unclear, although granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or granulocyte colony-stimulating factor (G-CSF) released by tumor cells have been involved. We studied whether CSF released by Ehrlich tumor (ET) may play a role. A comparative study was performed with two cell variants (ET and ET/0) growing in euthymic, *nude*, and SCID mice. Extramedullar hematopoiesis was assessed in the spleen by scoring organ enlargement, wheat germ agglutinin *ve+* cells, and interleukin 3-dependent granulocyte-macrophage colony-forming unit (GM-CFU). Both cell lines showed the same cytokine profile by reverse transcriptase polymerase chain reaction, including GM-CSF, G-CSF, and macrophage colony-stimulating factor (M-CSF); yet, only ET cells produced detectable colony-stimulating activity *in vitro*, mainly due to GM-CSF. No differences in tumorigenicity were noted between ET and ET/0 cells inoculated to normal or immunodeficient mice. An increase in extramedullar hematopoiesis, accompanied by neutrophilia and thrombocytosis, was associated with tumor progression irrespective of the cell line. A strong correlation was obtained between the increase in splenic GM-CFU and tumor mass ( $r = 0.96$ ,  $p < 0.0001$ ) that was independent on the tumor cell line, strain of mice, or stage of tumor development. The results point against CSF released by tumor cells and/or reactive host T cells as the only factors involved in the extramedullar hematopoiesis in this tumor model. The remarkable correlation between splenic GM-CFU and the tumor mass still suggests that a factor(s) of tumor origin may play a critical role. © 1999 International Society for Experimental Hematology. Published by Elsevier Science Inc.

**Keywords:** Cytokine—Tumor—Extramedullar hematopoiesis—Immunodeficient mice

### Introduction

It is well established that several human and murine nonhematologic neoplasms, including carcinomas, sarcomas, mesotheliomas, and melanomas, may produce constitutively colony-stimulating factor (CSF) [1–4] and CSF receptors [5,6]. This has been suggested to be a result of genetic aberrations in growth factor signaling pathways and/or a greater cytokine mRNA stability linked to the neoplastic transformation [7,8]. The physiologic function of CSF produced by tumor cells is not well understood, although both local and systemic effects have been implicated. At a local level, for example, granulocyte colony-stimulating factor (G-CSF)/granulocyte-macrophage colony-stimulating factor (GM-CSF) may promote tumor cell growth [9,10] or inhibition [11] by acting in an autocrine fashion and/or induce a host response leading to a decrease in tumorigenicity [12,13]. On the other hand, a number of systemic effects have been attributed to CSF of tumor origin in either human or experimental cancer (for a review, see Hall and Staszewski [14]). These effects include leukemoid reactions with neutrophilia [15], thrombocytosis [16], and hypercalcemia [17]. In experimental tumor models, a myeloid stimulation resulting in neutrophilia and extramedullar hematopoiesis with splenomegaly has been associated with G-CSF/GM-CSF production by tumor cells. In tumor bearers, this has been closely associated with the development of suppressor cells of myeloid lineage [18,19], accounting for a strong impairment of lymphoid responses and immunodeficiency, as also occurs in other conditions in which intense splenic hematopoiesis is occurring, e.g., graft-vs-host disease [20]. Although a number of studies found a relationship between tumor-derived CSF and these hematopoietic paraneoplastic syndromes [15–17], others failed to establish such a direct association [21–24]. For example, spleen enlargement and extramedullar hematopoiesis were described in tumors apparently lacking colony-stimulating activity (CSA) [21]. Additional mechanisms involving interactions among tumor cells, accessory cells, and lymphocytes have been suggested [23]. In this sense, endogenous factors that induce a he-

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matopoietic stress response, such as interleukin 1 (IL-1), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and IL-6, and/or factors from activated T lymphocytes, such as GM-CSF, IL-3, and IL-13, also might be involved.

We previously described that mice inoculated with Ehrlich tumor (ET) cells growing as a solid tumor develop marked splenomegaly, splenic hematopoiesis, and natural suppressor cells [19,25]. In line with other studies [26–28], we suggested that these phenomena were dependent on CSF released by ET cells [19], because constitutive production of CSF-like factors by ET cells had been described [29,30]. We realized, however, that an ET cell variant (ET/0) that also induced splenomegaly consistently lacked CSA. Taking advantage of both cell lines, we performed a comparative study with ET and ET/0 cells using euthymic, *nude*, and SCID mice to address these issues. Here we show that in vitro CSA derived from ET cells is mainly due to GM-CSF production. However, when ET and ET/0 were inoculated, no differences in the extramedullar hematopoiesis induced in tumor bearers were observed. This was also the case when T-cell-deficient (*nude* and SCID) mice were used instead of normal mice. However, a strong relationship was found between the increase in splenic granulocyte-macrophage colony-forming unit (GM-CFU) in tumor bearers and their tumor mass.

## Material and methods

### Mice

Eight- to twelve-week-old male C57BL/6 and C57BL/6 *nude* mice were purchased from Bommice (Bomholtgaard, Denmark). Eight- to ten-week-old male SCID mice were obtained from Centro de Biología Molecular (Madrid, Spain).

### ET cell lines

ET is a low metastatic spontaneously occurring murine mammary carcinoma. The hyperdiploid strain of ET was maintained in our laboratory since 1984 as ascitic tumor by weekly intraperitoneal transfer of  $10^6$  tumor cells in C57BL/6 mice. ET cells were frozen in liquid nitrogen and eventually maintained in vitro as cell culture in RPMI 1640 medium containing 2 mM L-glutamine, 10 mM HEPES, penicillin-streptomycin, and 5% endotoxin-free fetal calf serum. ET cells used in this study were maintained in vitro by serial passages in culture flasks. ET/0 cells were derived from ascitic ET cells that were cloned in vitro by limiting dilution. Aliquots of recently derived ET/0 were stored in liquid nitrogen. All tumor cell lines were regularly tested for mycoplasma contamination by staining with HOESCHT 33258 and examination by fluorescence microscopy.

### In vivo tumor growth

Tumor cells growing exponentially were collected from culture flasks and washed twice with Hank's balanced salt solution (HBSS). Viable cells (trypan blue dye exclusion) were counted. Cells were inoculated intramuscularly ( $2 \times 10^5$ ) into the left groin of mice. Tumor size was estimated by calculating the tumor area as

the product of two perpendicular diameters measured with Vernier calipers. In some experiments, primary tumors were obtained from intramuscularly growing tumors. These tumors were excised, minced, and passed through a stainless-steel screen. After washing with HBSS, tumor cells were cultured as described earlier.

### ET and ET/0 conditioned media

Supernatants from ET and ET/0 cell cultures seeded at  $1 \times 10^6$  cells/mL were obtained after 48-hour culture. Cells were removed by centrifugation (600g for 10 minutes). The supernatants were concentrated 2 $\times$  or 6 $\times$  by ultrafiltration on 10,000-Da cutoff Amicon membranes, filtered through 0.22  $\mu$ m, and frozen at  $-80^\circ\text{C}$  until used.

### Assay for CSA

CSA was detected in conditioned media (CM) derived from each tumor cell line and primary tumors obtained from ET/0 tumor-bearing mice by clonogenic assays in semisolid agar. Briefly,  $10^5$  bone marrow cells obtained as described [31] were plated in triplicate on 35-mm Petri dishes in 1 mL of semisolid Iscove's modified Dulbecco medium containing 20% horse serum, 5 to 30% CM, and 0.3% agar (Bacto-Agar, Difco Laboratories, Detroit, MI). After 7 days of incubation ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ), colonies containing more than 50 cells were scored under magnification (Hund, Wetzlar, Germany). In these experiments, both negative (control medium) and positive (15% WEHI-3B CM) controls were included. In some experiments, the presence of inhibitors for colony formation was tested in ET/0 culture supernatants by admixing (v/v) with CM derived from ET, WEHI-3B, or L929 cells, with these latter two as sources of IL-3 and macrophage colony-stimulating factor (M-CSF), respectively. Some experiments were carried out using neutralizing antibodies (R&D Systems, Minneapolis, MN) to GM-CSF, G-CSF, M-CSF, and leukemia inhibitory factor (LIF) at 5  $\mu$ g/mL and SCF at 10  $\mu$ g/mL, taking into account the information given by the supplier.

### Cytokine measurement

Commercially available ELISAs were used to measure IL-1 $\alpha$  (Amersham, Buckinghamshire, England) and GM-CSF (R&D Systems) in CM and sera obtained from retroorbital plexus of 28-day tumor-bearing mice. According to the manufacturer's specifications, the level of detection was 10 and 11 pg/mL, respectively. G-CSF, GM-CSF, and M-CSF bioactivities were assayed on NFS-60, FDC-P1, and M-NFS-60 cell lines (ATCC, Rockville, MD) in dimethylthiazol-diphenyltetrazolium (MTT) proliferative assays. The specificity of each bioassay was checked by adding appropriate neutralizing antibodies at 5  $\mu$ g/mL (R&D Systems). Tumor growth factor  $\beta$  (TGF- $\beta$ ) was measured by assessing the inhibition of Mv1Lu cell proliferation.

### Reverse transcriptase polymerase chain reaction analysis

RNA was extracted from  $10^6$  ET and ET/0 cells as described [31]. One microgram of RNA was reverse transcribed into single strand cDNA with oligo(dT) priming and 15 units of AMV RT (Promega, Madison, WI). The single-stranded cDNA was used as template for gene-specific polymerase chain reaction (PCR) amplifications of mouse  $\beta$ -actin, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-6, IL-10, IL-11, LIF, TNF- $\alpha$ , TNF- $\beta$ , SCF, interferon  $\gamma$  (IFN- $\gamma$ ), TGF- $\beta$ , G-CSF, GM-CSF, and M-CSF by using commercially available 5' and 3' primers (Stratagene, La Jolla, CA, and Clontech, Palo Alto, CA). Amplifications were conducted using Amplitaq DNA polymerase (Perkin-Elmer, Branchburg, NJ) in a 9600 Perkin-Elmer thermal cycler: initial denaturation at  $94^\circ\text{C}$  (5 minutes),

annealing at 60°C (5 minutes), and 25 or 50 cycles of 90 seconds at 72°C, 45 seconds at 94°C, and 45 seconds at 60°C, with a final extension of 10 minutes at 72°C. Negative and positive controls were included in all experiments. PCR products were detected in 2% NuSieve agarose gel electrophoresis and their lengths compared those referred by the suppliers of the primers using DNA standards.

#### Measurement of splenic hematopoiesis

Extramedullar hematopoiesis was measured in the spleen of tumor bearers by assessing splenomegaly, GM-CFU, and the numbers of wheat germ agglutinin (WGA) ve+ cells. Splenomegaly was estimated by weighting the spleen. GM-CFU were counted in semisolid agar cultures as described earlier, using  $10^6$  spleen cells with or without 15% WEHI-3B as the source of IL-3. GM-CFU increase over control mice, rather than absolute GM-CFU numbers, also was used for comparison among different strains of mice [32]. In some experiments, colonies were fixed with 5% glutaraldehyde (5 minutes) and methanol (overnight), air dried, stained (Wright), and visualized under microscopy. WGA ve+ cells were detected by flow cytometry as described later.

#### Blood counts

Blood samples were obtained from the retroorbital plexus at different times of tumor development. Blood cells were counted in a Coulter (Hialeah, FL) counter. Differential counts of leukocytes were made on 200 white cells from Wright-Giemsa-stained blood smears.

#### Flow cytometry

Flow cytometry was done using a FacScan (Becton Dickinson, Mountain View, CA). Spleen and bone marrow cells were isolated and erythrocytes lysed according to standard procedures. Cell suspensions ( $10^6$  cells/reaction) were incubated (30 minutes at 4°C) with 1 µg/mL of fluorescein isothiocyanate (FITC)-conjugated WGA (Sigma, St. Louis, MO) or 5 µg/mL of FITC-anti Mac-1 (PharMingen, San Diego, CA) in HBSS containing 2% fetal calf serum.

#### Immunofluorescence on spleens

Spleens from tumor-bearing or control mice were removed, cut into pieces that were placed in Tissuetek compound (Miles Inc., Elkhart, IN), and frozen in liquid nitrogen. Cryostat sections (8–10 µm) were incubated (45 minutes) with a dilution of A10, a monoclonal IgM antibody recognizing ET surface carbohydrates [33]. After a washing step, sections were treated (45 minutes) with a secondary FITC-labeled F(ab')<sub>2</sub> antibody to mouse µ chain containing 1% Evan's blue and examined, after a final washing, under a fluorescence microscope. Adjacent sections to those analyzed by A10 were stained with hematoxylin and eosin.

#### Statistical analysis

Data were analyzed using Microstat, an Ecosoft computer program. Experimental differences over the controls were assessed by Student's *t*-test. Probability values greater than 0.05 were considered nonsignificant. In all cases, at least three independent experiments were conducted to warrant that the results were representative.

## Results

#### Characterization of the cytokine profile associated with ET and ET/0 cells

Table 1 lists a representative experiment testing the presence of CSA in supernatants derived from ET and ET/0 cell cultures. As shown, whereas supernatants from ET cells

Table 1. Colony-stimulating activity in conditioned medium derived from ET and ET/0 cells

CM source*	Percent†	No. of CFU‡
None	—	0
WEHI-3B cells	20	94 ± 8
ET cells	10	8 ± 4
ET cells	20	24 ± 7
ET cells	30	39 ± 9
ET cells	40	32 ± 10
ET/0 cells	10	0 ± 0
ET/0 cells	20	1 ± 1
ET/0 cells	30	0 ± 0
ET/0 cells	40	0 ± 0
ET/0 cells (6×)	20	1 ± 1

\*Supernatants from 48-hour cell cultures at an initial cell concentration of  $10^5$  cell/mL.

†Percentage of conditioned medium (CM) used in the clonogenic assay.

‡Number of colony-forming units (CFU) scored in a clonogenic assay using  $10^5$  bone marrow cells (mean of triplicates ± SD).

induced CFU in a roughly linear fashion up to 30 % (v/v), those derived from ET/0 cells were fully unsuccessful. Because the in vitro ET cell growth rate was estimated at 20% faster than ET/0 as measured by thymidine uptake (data not shown), experiments also were performed after ET/0 CM concentration (6×); however, similar results were obtained (Table 1). To determine if ET/0 cells were producing inhibitors of CFU induction, clonogenic assays were performed with CM derived from ET cells, WEHI-3B, or L929 cells (as source of CSA, IL-3, and M-CSF, respectively), admixed with that of ET/0 cells. As shown in Table 2, no differences were noted in these mixtures in any case, ruling out in ET/0 supernatants the presence of putative suppressor factors for colony formation.

The next series of experiments were conducted to characterize the CSA associated with ET cells, using ET/0 for comparison. Figure 1 shows the mRNA pattern detected by reverse transcriptase (RT)-PCR of hematopoietic relevant

Table 2. Conditioned medium from ET/0 cells lacks inhibitors for colony formation

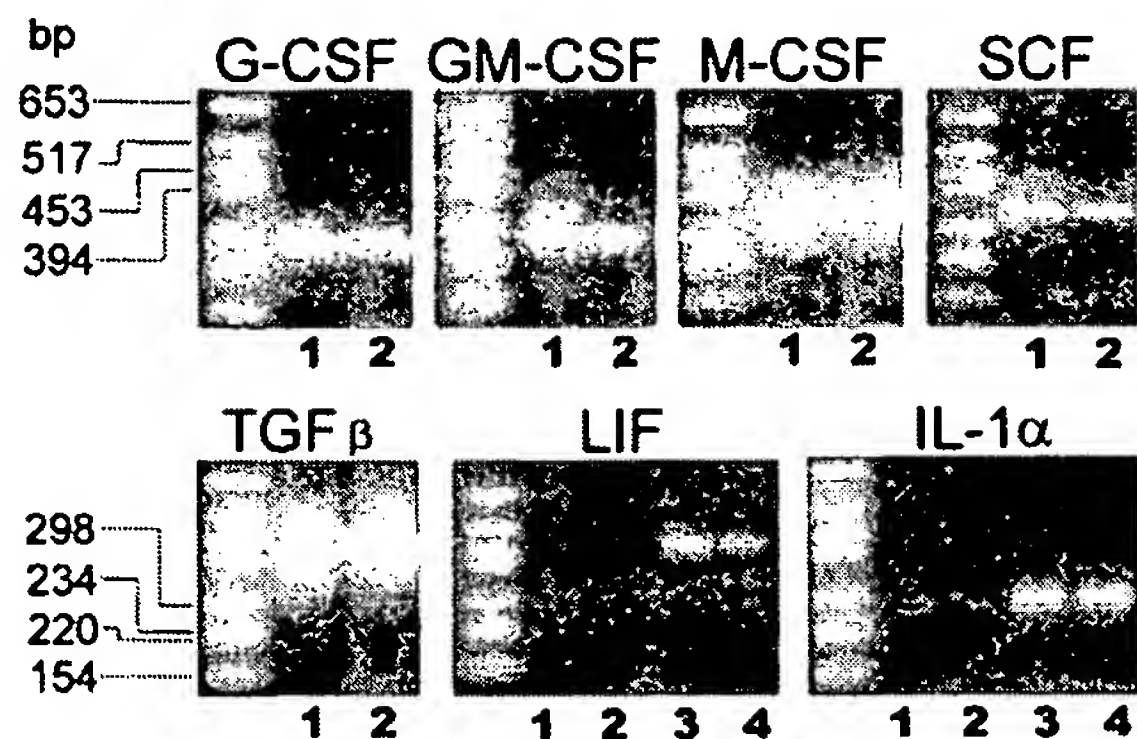
CM source*	CM from ET/0 (%)†	No. of CFU‡
WEHI-3B cells	—	91 ± 7
WEHI-3B cells	15	87 ± 8
WEHI-3B cells	30	85 ± 10
L929 cells	—	82 ± 11
L929 cells	15	90 ± 13
L929 cells	30	77 ± 10
ET cells	—	30 ± 5
ET cells	15	27 ± 6
ET cells	30	33 ± 7

CM = conditioned medium.

\*As in Table 1. The final percentages were 20% (WEHI-3B), 15% (L929), and 30% (ET).

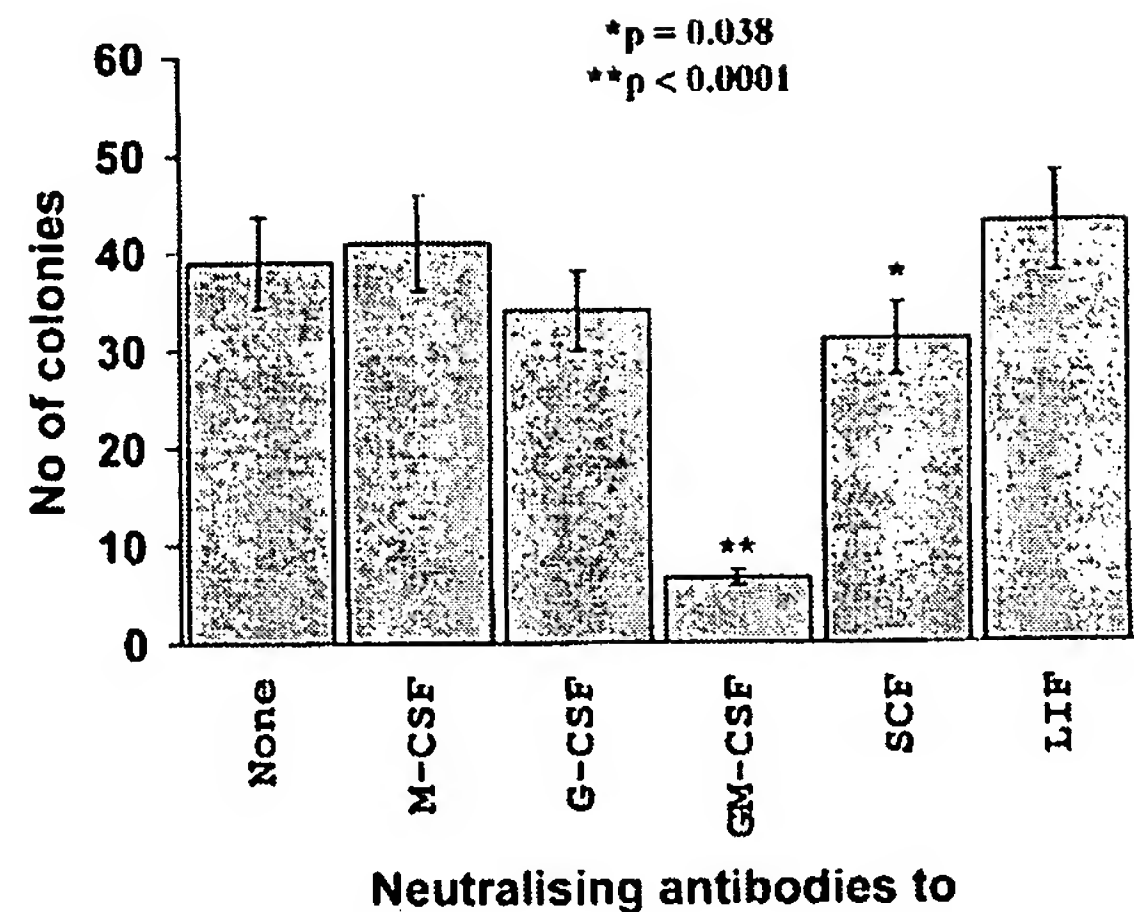
†Percentage of ET/0 admixed.

‡Number of colony-forming units (CFU) scored in a clonogenic assay using  $10^5$  bone marrow cells (mean of triplicates ± SD).



**Figure 1.** Cytokine mRNA expression by ET and ET/0 cells as detected by RT-PCR. cDNA derived from ET (lines 1 and 3) or ET/0 (lines 2 and 4) was amplified with specific primers for 25 (lines 1 and 2) or 50 cycles (lines 3 and 4). PCR amplification fragments were separated in agarose gels and visualized under ultraviolet light.

CSF and cytokines displayed by either ET or ET/0 cells. Both cell lines expressed the same cytokine profile, being detectable G-CSF, GM-CSF, M-CSF, SCF, and TGF- $\beta$  (25 cycles) and LIF and IL-1 $\alpha$  (50 cycles). No messages were detected (50 cycles) for IL-1 $\beta$ , IL-2, IL-3, IL-6, IL-10, IL-11, TNF- $\alpha$ , TNF- $\beta$ , or IFN- $\gamma$  (not shown). The same pattern was obtained from three separate RNA extractions performed at different culture times. Then, M-CSF, G-CSF, GM-CSF, IL-1 $\alpha$ , and TGF- $\beta$  were measured by ELISA and/or bioassays in ET and ET/0 CM (Table 3). As shown, G-CSF and GM-CSF were detectable in cultures derived from ET cells, with mean estimated secretion rates of 11 and 98 pg/10<sup>6</sup> cells/48 hours, respectively. Neutralizing experiments (Fig. 2) indicated that most of the CSA found in ET cell culture CM was dependent on GM-CSF, although a slight but significant decrease also was observed on SCF neutralization. In contrast, no significant inhibition was seen when neutralizing G-CSF. Taken together, these re-



**Figure 2.** Effect of cytokine neutralization on the colony-stimulating activity associated with ET cells. Clonogenic assays were performed with bone marrow cells in semisolid agar using ET cell-conditioned medium (30% final volume) in the presence or absence of cytokine neutralizing antibodies at 5 or 10  $\mu$ g/mL (SCF). Results are given as the mean  $\pm$  SD of triplicate cultures. The *p* values correspond to statistical differences as compared with control cultures (without adding antibodies).

sults indicated that ET tumor cells released functional CSF, mainly GM-CSF, whereas ET/0 tumor cells did not.

#### Effect of ET and ET/0 cells growing in vivo

ET and ET/0 cells ( $2 \times 10^5$ ) were inoculated intramuscularly into the left groin of C57BL/6 mice. As shown in Figure 3, tumor formation was evident, without significant differences between both cell lines at any stage of tumor development (35 days of follow-up). As the tumor grew, both spleen enlargement and splenic GM-CFU increased irrespective of the tumor cell line inoculated (Fig. 4). Such an increase was significant ( $p < 0.01$ ) as early as day 14 after tumor challenge (spleen weight) and day 7 (GM-CFU), as compared with control mice. Figure 5 shows that the number of WGA ve+ cells in spleens of ET and ET/0 increased from a basal <10% to about a 30% at day 35 of tumor de-

**Table 3.** Measurement of cytokines in conditioned medium from ET and ET/0 cells

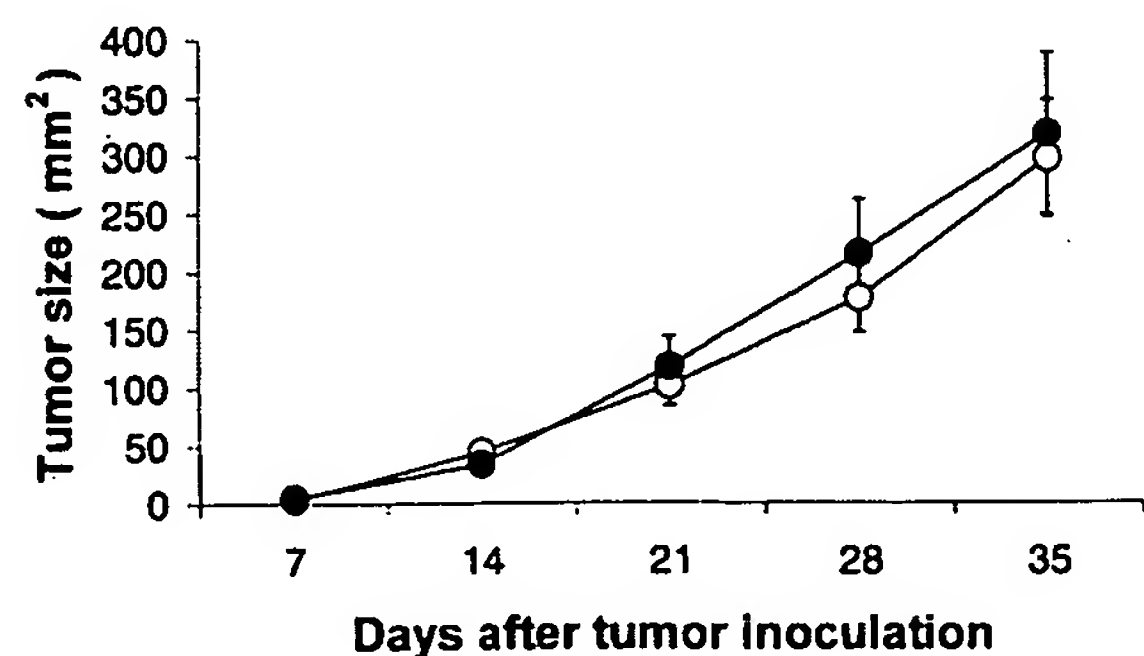
Cytokine	CM source*	
	ET cells	ET/0 cells
GM-CSF†	98 $\pm$ 8	<11
G-CSF†	11 $\pm$ 3	<9
M-CSF‡	<25	<25
IL-1 $\alpha$ †	<10	<10
TGF- $\beta$ ‡	<50	<50

CM = conditioned medium.

\*As in Table 1.

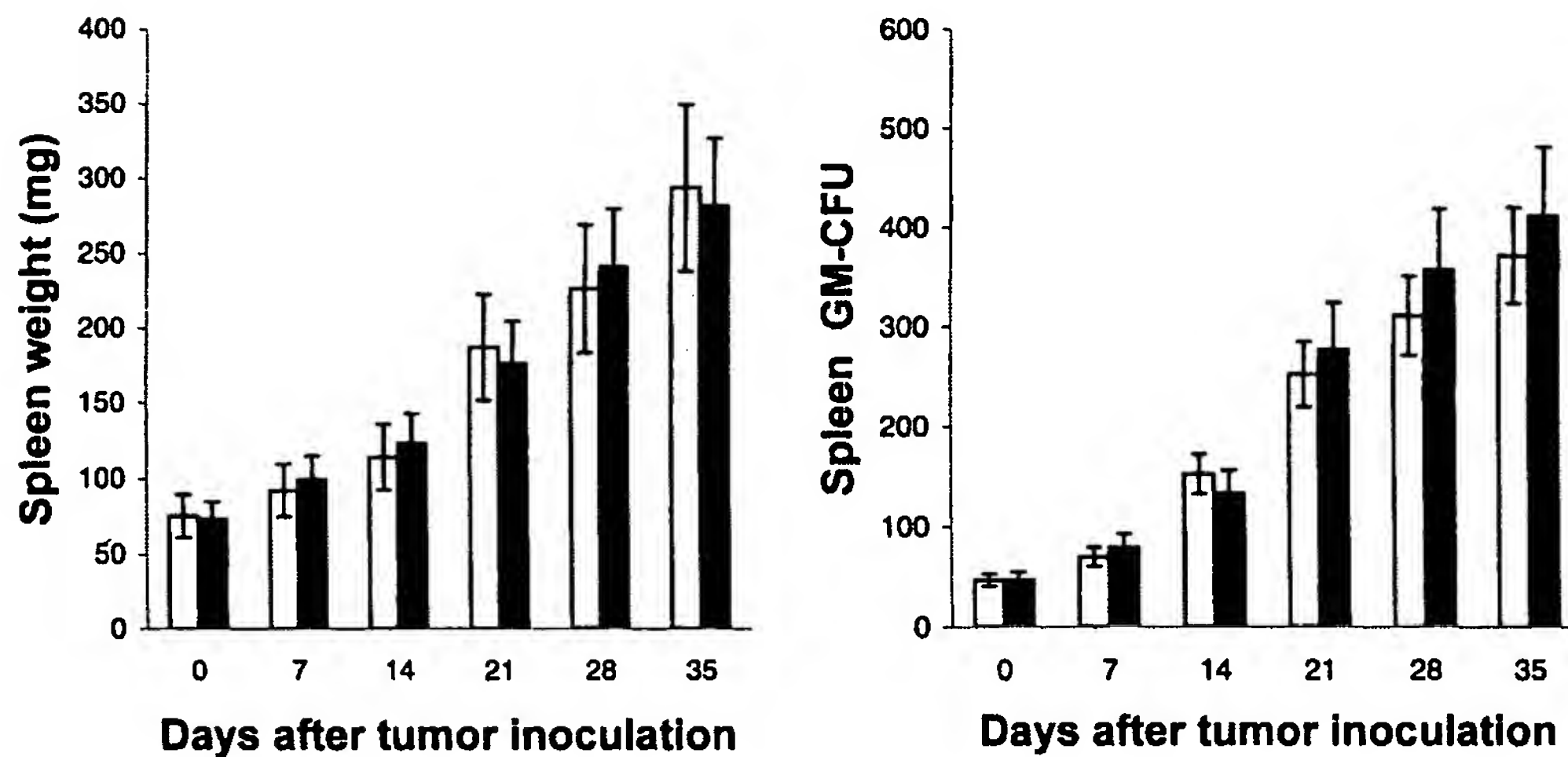
†Measured by ELISA.

‡Measured by bioactivity as described in the Material and methods.



**Figure 3.** Tumor growth rates in C57BL/6 mice inoculated with ET (open circles) or ET/0 (solid circles) cells ( $2 \times 10^5$ ) intramuscularly in the left groin. Results are given as the mean  $\pm$  SD for eight mice.





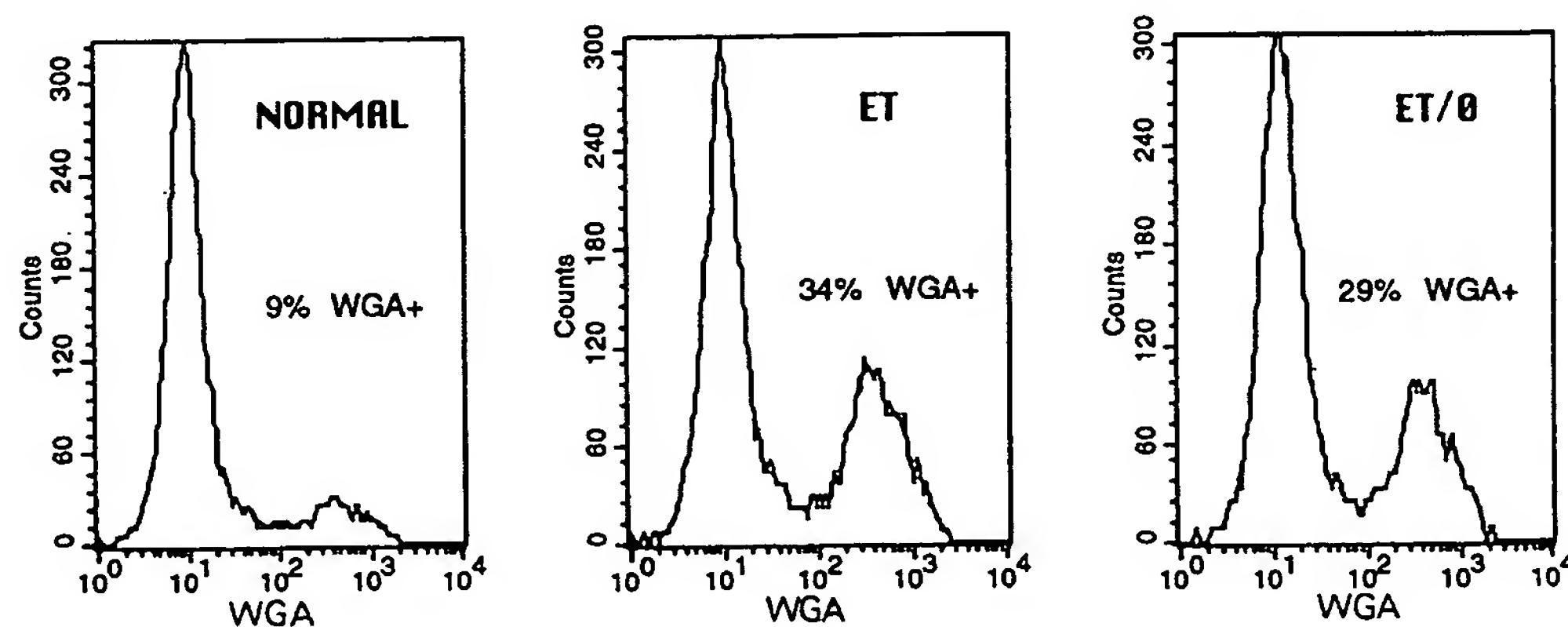
**Figure 4.** Splenomegaly and splenic GM-CFU (number per  $10^6$  spleen cells) induced in C57BL/6 mice inoculated with ET (open bars) or ET/0 (solid bars) cells ( $2 \times 10^5$ ) intramuscularly in the left groin. Results are given as the mean  $\pm$  SD for five mice sacrificed at different stages of tumor development.

velopment, whichever cell line was inoculated. In both ET- and ET/0-bearing mice (35 days), these phenomena were accompanied by blood neutrophilia ( $25.4 \times 10^3/\mu\text{L}$  vs  $8.9 \times 10^3/\mu\text{L}$  in controls) and thrombocytosis ( $204 \times 10^3/\mu\text{L}$  vs  $128 \times 10^3/\mu\text{L}$  in controls) as well as a strong myeloid stimulation in bone marrow ( $>90\%$  Mac-1 $^+$  vs 30–40% Mac-1 $^+$  in controls) (data not shown). As these results pointed against a role for CSF released by ET cells, we were prompted to rule out that CSF-producing ET/0 cell variants were being selected while growing in vivo. ET/0 cells obtained from two different tumors were checked as described earlier (Table 4). As shown, the results were similar to those previously found, indicating that ET/0 cells derived from these tumors still lacked such CSF production. On the other hand, the serum level of GM-CSF in tumor-bearing mice was under the detection limits of the assay (11 pg/mL) in all cases, and tumor-bearing sera were unable

to support NFS-60 and M-NFS 60 cell proliferation, and G-CSF- and M-CSF-dependent cell lines.

#### *Effect of ET and ET/0 cells growing in T-cell-immunodeficient mice*

Because of the results obtained, we assessed the involvement of host T cells in the extramedullar hematopoiesis observed in ET- and ET/0-bearing mice. Similar experiments were performed in T-cell-immunodeficient C57BL/6-*nude* or SCID mice. Figure 6 shows the effect of ET and ET/0 growing in these mice, as compared with normal C57BL/6 at two stages (days 14 and 28) of tumor development. There were significant differences in tumor growth related to the strain of mice tested. The tumor grew faster in SCID  $>$  *nude*  $>$  normal mice, but growth was not related to the tumor cell line inoculated. As shown in Figure 6, the increase



**Figure 5.** Induction of wheat germ agglutinin (WGA) ve+ cells in the spleen of C57BL/6 mice inoculated with ET or ET/0 cells ( $2 \times 10^5$ ) intramuscularly in the left groin after 35 days of tumor growth. Spleen cells (erythrocyte free) were analyzed by flow cytometry. Results are representative from six tumor-bearing mice assayed.

**Table 4.** Colony-stimulating activity and cytokine production in conditioned medium from ET/0 cells derived from solid tumors

Assay	CM source*		
	ET/0 cells (a) <sup>†</sup>	ET/0 cells (b) <sup>†</sup>	ET cells
CSA <sup>‡</sup>	0 ± 0	0 ± 0	28 ± 5
GM-CSF	<11	<11	105 ± 11
G-CSF	<9	<9	ND
Cytokines <sup>§</sup>			
M-CSF	<25	<25	ND
IL-1α	<10	<10	ND
TGF-β	<50	<50	ND

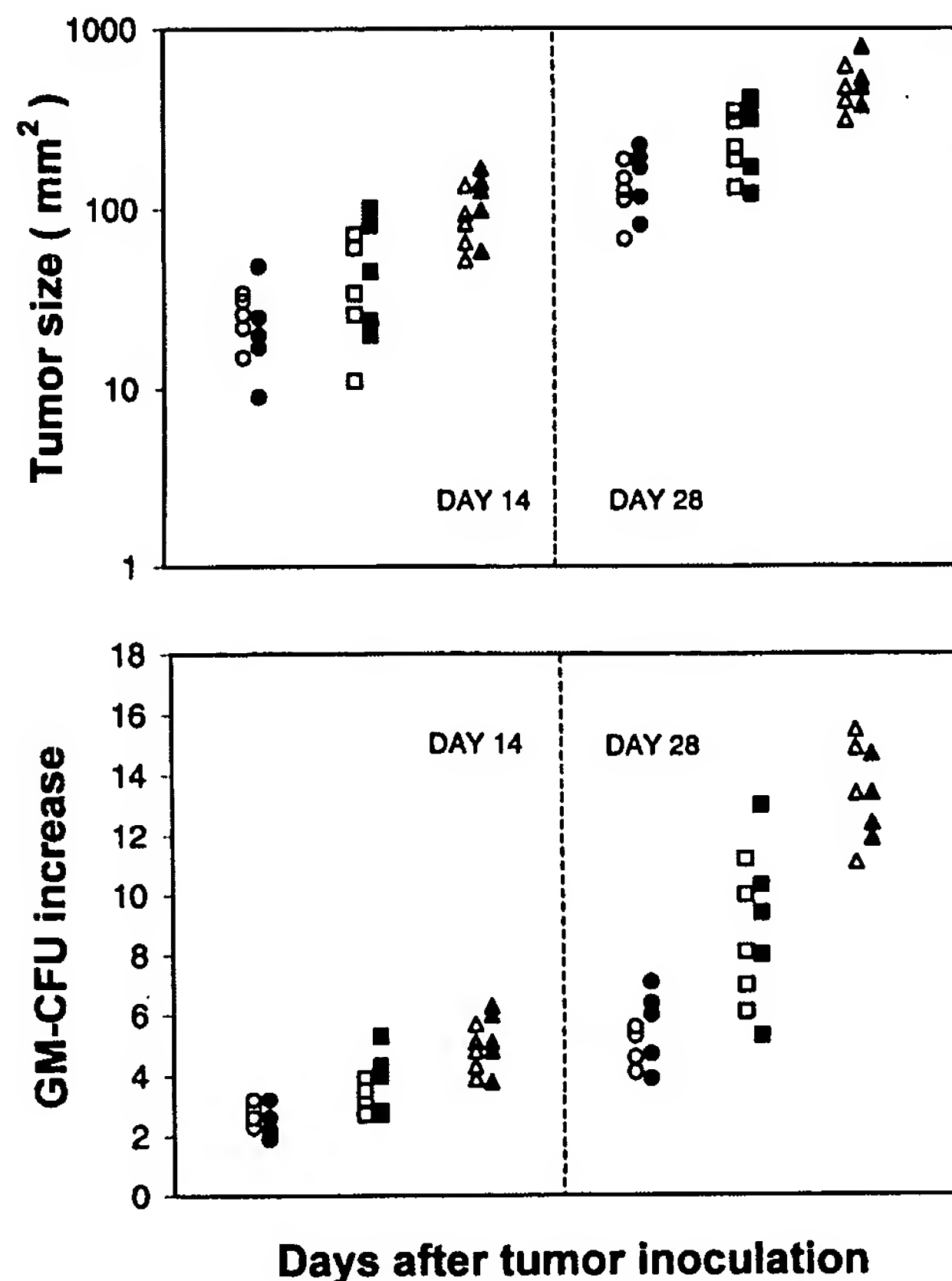
\*As in Table 1.

<sup>†</sup>Derived from ET/0 cells growing in two C57BL/6 mice (a, b) at day 35 of tumor development.<sup>‡</sup>Assayed in clonogenic assays as in Table 1 using 30% of conditioned medium (CM) (final percentage). Data are the number of colony-forming units (mean of triplicates ± SD).<sup>§</sup>As in Table 3. Data are expressed in pg/mL (mean of triplicates ± SD).

in splenic GM-CFU was greater in SCID > *nude* > normal mice; yet, again the increase was irrespective of the tumor cell challenged. Because a direct relationship between tumor mass and splenic GM-CFU seemed evident, both variables were plotted together for the same mice (Fig. 7). As shown, a strong correlation was found ( $r = 0.96$ ,  $p < 0.0001$ ), irrespective of using for computation all three different strains of mice, both tumor cell variants, or two different stages of tumor development. Given this remarkable correlation, the possibility of metastatic tumor cells accounting for spleen colonies was considered, even though no splenic CFU were scored in the absence of IL-3 and that ET cells hardly metastasize in spleen [19]. Figure 8 shows cryostat sections of spleens from SCID mice stained with A10, a monoclonal antibody recognizing ET cell surface carbohydrates [33]. In tumor bearers, a punctate pattern of A10 reactivity was detected, mostly concentrated outside the periarteriolar cell sheath, i.e., marginal zone. This reactivity was much weaker and different from the strong and linear stain on ET cells (not shown), pointing against the presence of metastatic cells but to A10-reactive tumor-derived carbohydrates bound to marginal host macrophages. Nevertheless, to ensure the cell origin of splenic CFU scored in ET-bearing SCID mice, these colonies were analyzed microscopically after dye staining. The results from three mice (30 days of tumor development) indicated that virtually all colonies contained polymorphonuclear and mononuclear cells (Fig. 9), and, therefore, were typed as granulocyte/macrophage.

### Discussion

The data presented herein indicate that, in common with several human and murine nonhematologic tumors, ET cells release CSF constitutively. Previously, ET cells had been



**Figure 6.** Tumorigenicity and splenic GM-CFU-inducing ability of ET (open symbols) or ET/0 (solid symbols) cells ( $2 \times 10^5$ ) inoculated intramuscularly in the left groin of C57BL/6 (circles), *nude* (squares), or SCID (triangles) mice. GM-CFU increase was calculated over the mean number of splenic GM-CFU obtained from their respective control mice (without tumor challenge). Each symbol represents data scored from individual mice.

described as releasing factors that promoted the differentiation of myeloid M1 cells [30], a property shared by G-CSF, IL-6, and LIF [34]. Here, CSF produced by ET cells were identified as GM-CSF and G-CSF by RT-PCR, bioactivity, and/or protein. Most of the CSA detected in ET CM was prevented by GM-CSF neutralization. SCF was detectable by RT-PCR and appeared to be involved in ET-derived CSA as well, although its relative contribution to this activity was, like G-CSF, scarce. In contrast, ET/0 CM lacked any detectable CSA, even at  $6 \times$  concentration. Moreover, no synergistic effects were noted between ET/0 and WEHI-3B, L929, or ET CM in any clonogenic assay, suggesting that SCF or other factors acting on early progenitors are unlikely to be released by ET/0 cells in vitro. The ET/0 cell line was a spontaneously arising ET cell variant obtained in this laboratory after ET cell cloning and selected because their lack of CSA. This lack was not due to the putative presence of inhibitors for colony formation [35] nor to synergistic suppression [36]. GM-CSF was undetectable by ELISA in ET/0 CM. Moreover, ET/0 CM could not support

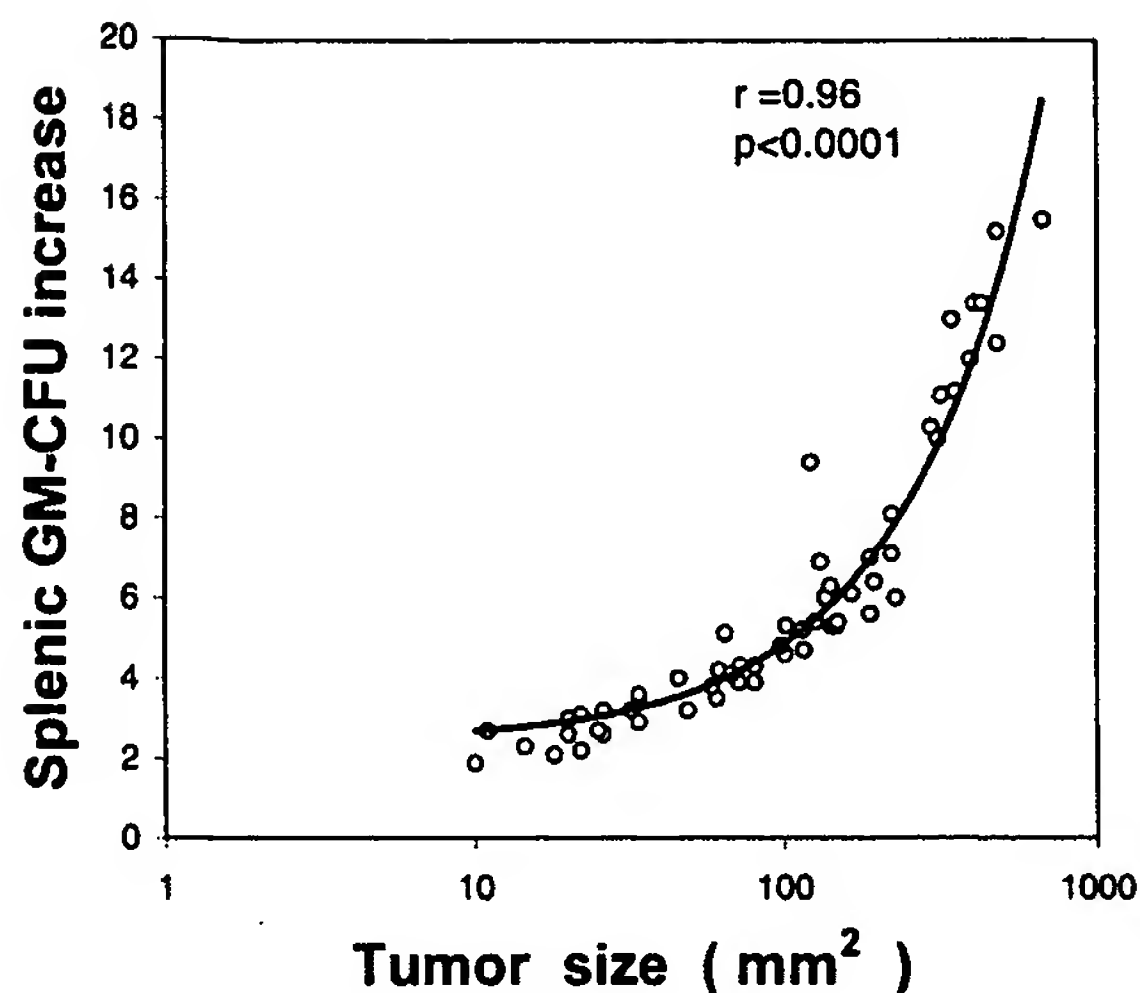


Figure 7. Relationship between the increase in splenic GM-CFU and tumor size. Plotted data were from individual mice corresponding to the same experiment shown in Figure 6.

the proliferation of G-CSF-, M-CSF-, and/or GM-CSF-dependent cell lines. Nevertheless, the transcription of CSF genes was similar in ET and ET/0 cells as detected by RT-PCR, suggesting that, in ET/0 cells, GM-CSF and G-CSF were transcribed but not translated to protein. This also appeared to be the case for other cytokines such as M-CSF, IL-1 $\alpha$ , and TGF- $\beta$ , which were transcribed but remained undetectable in cell culture supernatants. The differential cytokine production by these tumor cell variants fit with the notion that, although cytokine mRNA stability is enhanced on transformation [37], the efficiency of translation is variable among tumor cells [38]. Similar differences were reported by testing the cytokine expression and/or secretion profiles of individual tumor cells derived from the same neoplasm [39,40].

When inoculated, the tumor growth rates of ET and ET/0 were remarkably similar, irrespective of the differences in CSF released in vitro. This in vivo behavior may be in disagreement with the lower tumorigenicity expected for tumor cells releasing CSF, as observed with cytokine engineered tumor cells secreting G-CSF [12] and/or GM-CSF [41]. However, because a relationship between the amount of cytokine released and tumor impairment exists [41], it is possible that the amount of GM-CSF (98 pg/10<sup>6</sup> cells/48 hours) and G-CSF (11 pg/10<sup>6</sup> cells/48 hours) released by ET cells might not be large enough to decrease their tumorigenicity. Moreover, the possibility that ET cells produce immunosuppressive factors subverting the host's immune response [42,43] and therefore counteracting the effect(s) of CSFs should be considered.

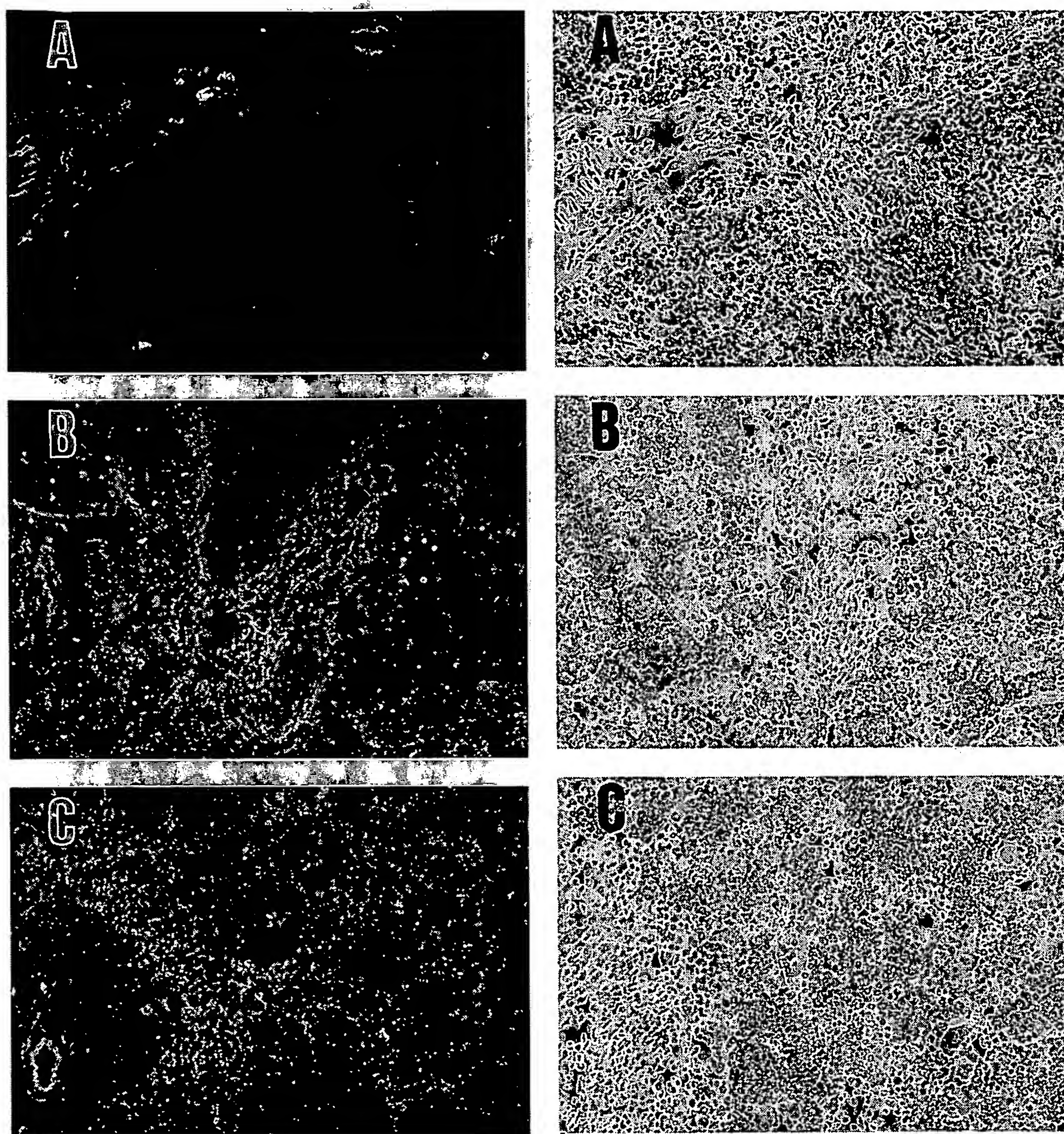
When C57BL/6 mice were inoculated with the ET or ET/0 cell line, similar spleen enlargement, splenic myelopoiesis, and blood neutrophilia were recorded. Spleen enlargement was due to a higher cellularity associated with the presence

of WGA<sup>+</sup> cells, which is consistent with an increase of hematopoietic progenitors [44]. This increase also was seen by counting the numbers of splenic CFU in response to IL-3, i.e., committed myeloid precursors GM-CFU, but also in committed erythroid precursors CFU-E and in day 7 CFU-S (data not shown). Although this redistribution of myeloid progenitor cells closely resemble the effects of recombinant G-CSF and/or GM-CSF administration [45], the present results point against an effect based merely on the release of GM-CSF/G-CSF by tumor cells, at least in this tumor system. In this sense, the serum levels of GM-CSF and G-CSF were undetectable in ET-bearers.

A number of experimental tumor models, including ET, have been described to induce myeloid stimulation, neutrophilia, spleen enlargement, and development of splenic suppressor cells. However, these phenomena are not universal in tumor bearers. They have been associated mostly with extramedullar hematopoiesis and related to constitutive production of CSF by tumor cells [27,28]. The present results, however, may be consistent with the strong neutrophilia observed in other tumor models otherwise releasing low amounts of CSF [22]. Moreover, a converse report showing that a tumor cell variant lacked neutrophilia, while still releasing CSA [21], may point in the same direction. Nevertheless, single hematopoietic growth factors appear to act in vivo in concert with low amounts of other regulators, exerting net effects on hematopoietic cell populations [46]; therefore, we cannot formally exclude the possibility of subliminal amounts of tumor-derived CSF whose genes were at least transcribed in both cell lines.

The involvement of host T cells on tumor-associated splenic myeloid stimulation was considered, as T-cell-derived cytokines are thought to play a major role in reactive hematopoiesis. On activation, T cells may drive the expansion of hematopoietic cells during stress-induced responses by releasing GM-CSF and IL-3 [47] and develop extramedullar hematopoiesis through IL-13 production [48]. Nevertheless, when ET or ET/0 was grown in *nude* and SCID mice, induction of splenic GM-CFU was seen, suggesting that T-cell function is not critical for this task in our tumor bearers. Moreover, these results support those obtained in euthymic mice, because no differences were noted between ET and ET/0 cells. These findings are in line with studies indicating that T lymphocytes may not be required to induce reactive myelopoiesis in the adult spleen following various treatments such as irradiation or lipopolysaccharide (LPS) inoculation [49]. A report indicating that hematopoiesis occurs in emergency situations, even in the complete absence of IL-3, GM-CSF, and IL-5, provides additional support [50]. Of note, these results show that the increase of GM-CFU scored in T-deficient mice were even higher than in normal mice. This was remarkable, because in these mice both ET and ET/0 cell lines grew faster and thus a correlation was found between GM-CFU increase and the tumor mass. The possibility of metastatic tumor cells being scored





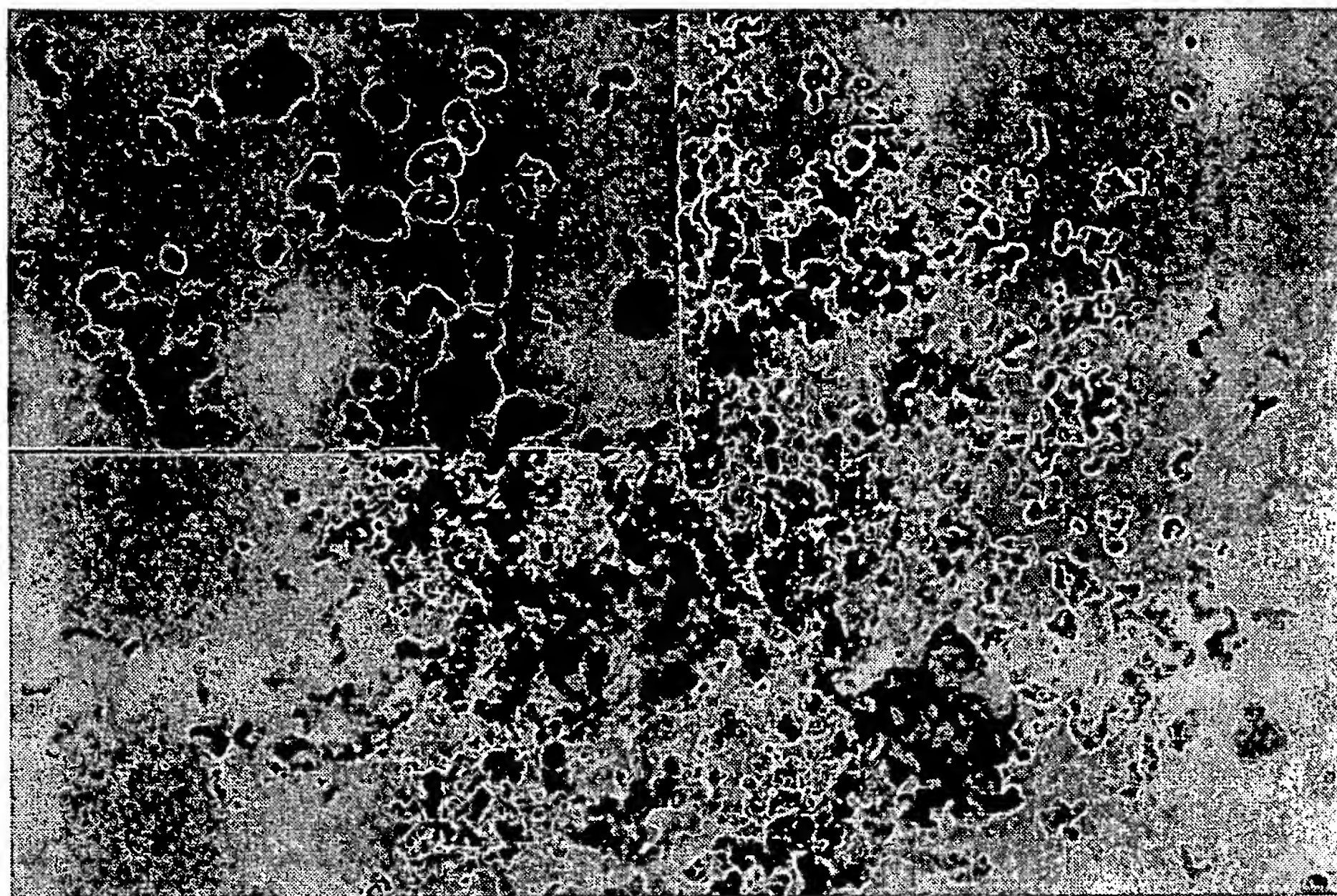
**Figure 8.** A10 reactivity (left) and hematoxylin-eosin staining (right) on adjacent cryostat sections of spleens from control (A), ET-bearing (B), and ET/0-bearing (C) SCID mice (28 days of tumor development). A10 reactivity was analyzed by indirect immunofluorescence on samples counterstained with Evan's blue, which accounts for the red (gray) background. Note the A10 reactivity outside the periarteriolar cell cuff and the presence of megakaryocytes (hematoxylin-eosin) in tumor bearers (original magnification  $\times 200$ ).

as CFU was ruled out because 1) no ET cells were detected in the spleens; 2) no CFU were scored in the absence of IL-3; and 3) almost all colonies contained polymorphonuclear cells on staining.

Because neither tumor-derived CSF nor a tumor-reactive host T response appears responsible for the extramedullar hematopoiesis observed in ET-bearing mice, the question is what other mechanism(s) might be. Because a strong relationship between extramedullar hematopoiesis and the tumor mass suggests an effect that is dependent on a product(s) re-

leased by the tumor cells. In this regard, ET cells, as many other tumors, release a wide variety of substances. Among these, carbohydrates [25,51] and vascular endothelial growth factor (VEGF) [52] warrant attention. ET cell surface carbohydrates, proteoglycans and/or mucins, are present in circulation in increasing amounts as the tumor grows [25] and may deposit in hematopoietic tissues as seen here for A10-reactive carbohydrates. In this sense, the possibility exists of these highly glycosylated structures enhancing extramedullar hematopoiesis, as described for polysaccharides inducing





**Figure 9.** Representative colony-forming unit derived from spleen cells of tumor-bearing SCID mice (28 days of tumor development). Seven-day colonies obtained in semisolid agar containing IL-3 were fixed, air dried, and stained with Wright. Note the abundant polymorphonuclear cells (original magnification  $\times 100$ ; insert: original magnification  $\times 400$ ).

splenic GM-CFU in a dose-related manner [53,54]. Of note, ET-derived A10-reacting carbohydrates were shown to bind to the macrophage cell surface *in vitro* [55] and likely to splenic marginal macrophages *in vivo* (Fig. 8). This macrophage population expresses the sialic acid receptor sialoadhesin [56] and, because A10 recognizes tumor sialomucins [57], a binding through this receptor should be considered. If this were the case, increasing amounts of circulating carbohydrates of tumor origin might result in a functional blockade of this receptor in bone marrow stromal macrophages, inhibiting their sialoadhesin-dependent adhesion with hematopoietic progenitor cells [58] and, therefore, disturbing the normal hemopoiesis. Alternatively, ET-derived proteoglycans containing heparan sulfate [59] might stimulate extramedullar hematopoiesis through presentation of endogenous growth factors [60]. Clearly, more work is necessary to elucidate these issues and to explain the putative role of tumor-derived carbohydrates in these paraneoplastic phenomena.

A recent report [61] addressing the *in vivo* effect of VEGF may provide some clues about the origin of extramedullar hematopoiesis in tumor models, greatly supporting our contention. Although VEGF plays a critical role in tumor neoangiogenesis, Gabilovich et al. [61] showed that it also induces dramatic hematopoietic changes when given in a continuous long-term infusion to normal mice, at pathologically relevant concentrations *in vivo*. These changes include intense neutrophilia and splenomegaly with a strong increase in GM-CFU and megacaryocyte numbers, as we showed here in ET bearers (Fig. 8). It should be noted that the concentrations of VEGF used in the mentioned study

can be readily achieved in tumor bearers [61] and that ET cells are recognized as high producers of this factor [52], which was confirmed by ELISA (R&D Systems) in both ET and ET/0 cell lines. Whether the hematopoietic changes induced in ET-bearing mice can be explained solely by VEGF production requires further studies. That tumor-derived VEGF binds to sulfated proteoglycans [52] may point to tumor-derived carbohydrates playing some role as well.

#### Acknowledgments

This work was supported by grant SAF 96/378 to J.L.S. We thank Dr. Bueren (CIEMAT, Madrid, Spain) for technical assistance and Dr. Kusmartsev (Tomsk, Russia) for helpful discussion. Special thanks to Dr. Palacín (CBM, Madrid, Spain) for providing us with the SCID mice.

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